EXHIBIT 2

ICOS is critical for T helper cell–mediated lung mucosal inflammatory responses

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We examined the requirement for and cooperation between CD28 and inducible costimulator (ICOS) in effective T helper (T_H) cell responses in vivo. We found that both CD28 and ICOS were critical in determining the outcome of an immune response; cytolytic T lymphocyte-associated antigen 4-immunoglobulin (CTLA-4-Ig), ICOS-Ig and/or a neutralizing ICOS monoclonal antibody attenuated T cell expansion, T_H2 cytokine production and eosinophilic inflammation. CD28-dependent signaling was essential during priming, whereas ICOS-B7RP-1 regulated T_H effector responses, and the up-regulation of chemokine receptors that determine T cell migration. Our data suggests a scenario whereby both molecules regulate the outcome of the immune response but play separate key roles: CD28 primes T cells and ICOS regulates effector responses.

Optimal T cell expansion is regulated by signals delivered through the T cell receptor (TCR) and a number of costimulatory molecules[1,2]. CD28 ligation on antigen-inexperienced precursor T helper (T_HP) cells by its counter-receptors B7-1 and B7-2, which are expressed on dendritic cells, play a crucial role in initial T cell priming. interleukin 2 (IL-2) production and cycle cell progression[3,4]. Recently primed T_HP cells migrate to the T cell zone of the B cell follicle where they encounter antigen-specific B cells that have also migrated to this area[5]. T cells deliver, primarily through CD40, a signal for B cell expansion. Primed T cells in turn receive additional signals from B cells that regulate T_H function. CD28-mediated T cell expansion is opposed by cytolytic T lymphocyte–associated antigen 4 (CTLA-4)[6], which also binds both B7-1 and B7-2; its function is to attenuate the T cell expansion and cytokine production of recently activated T cells. The importance of CTLA-4–mediated suppression is illustrated in gene-deficient mice, which exhibit autoimmune disease characterized by marked T cell expansion and death within 3–4 weeks[6].

The inducible costimulatory molecule (ICOS)[7] is the third member of the CD28 superfamily and, like CTLA-4, is expressed on antigen-primed T cells[8,9]. ICOS binds its own B7 family member, B7RP-1[10] or B7h[11], on B cells and macrophages[11]. ICOS can induce CD28-independent T cell expansion and selective cytokine production[9,11]. Similarly, signaling though CD28 provides a signal for T_H2 cytokine production in vitro[12]. It has been proposed that T_H2 responses are more dependent on CD28-mediated costimulation than T_H1 responses, although this remains somewhat controversial[2]. Data from ICOS-deficient mice have shown that this molecule plays a critical role in T–B cell interactions, is essential for germinal center formation and humoral immune responses and delivers a key signal for IL-4, but not interferon-γ (IFN-γ), production[14,16]. However, the contribution of these different costimulatory effects in regulating T cell function during inflammatory processes remains unknown.

Effective immune responses require not only the appropriate costimulatory signals necessary for optimal cytokine production but also the coordinated migration of T cells into the lymph nodes and target tissues, a process that is critically regulated by chemokines and their receptors[17]. T_H effector cells can be divided not only on the basis of their ability to produce different cytokines but also on the differential expression of chemokine receptors. T_H1 cells express the chemokine receptors CXCR3 and CCR5, whereas T_H2 cells express CCR3, CCR4 and CCR8[18,19]. This differential chemokine receptor expression may play an important role in determining whether T_H1 or T_H2 cells accumulate at sites of delayed-type hypersensitivity or allergic inflammation. The relationship, however, between costimulatory signals and chemokine receptor–mediated T cell migration remains largely unexplored.
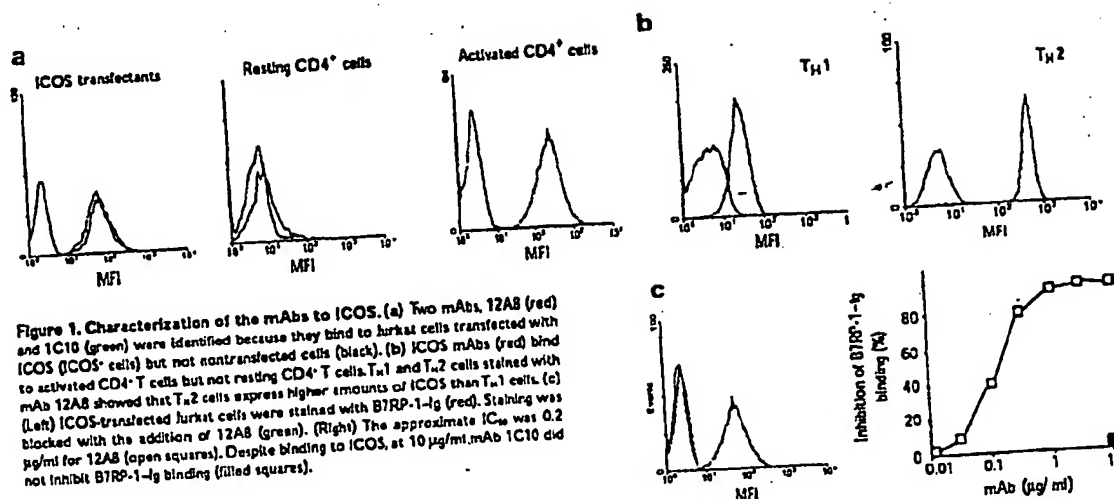
We have investigated the role of ICOS and CD28 in regulating T_H2-mediated mucosal inflammatory responses. Our data shows that CD28 is critical during priming but does not contribute to recall responses once effector T_H2 cells have been generated. In contrast, ICOS plays the predominant role in regulating T_H2 effector cell activation. ICOS-mediated signaling contributes to the inflammatory response not only through the regulation of IL-4 but by providing a signal for up-regulation of the chemokine receptors CCR3, CCR4 and CCR8. The findings reported here indicate that strategies aimed at inhibition of ICOS may represent a therapeutic target during allergic inflammatory responses and other disorders characterized by inappropriate T cell activation.

Results
Generation of neutralizing mAbs to ICOS
Monoclonal antibodies (mAbs) to ICOS, 12A8 and 1C10, were used to stain ICOS-transfected Jurkat cells (ICOS⁺ cells), which were assessed by flow cytometry; they did not stain untransfected Jurkat cells (Fig. 1a). Activated, but not resting, CD4⁺ T cells were also stained by 12A8

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Figure 1. Characterization of the mAbs to ICOS. (a) Two mAbs, 12A8 (red) and 1C10 (green) were identified because they bind to Jurkat cells transfected with ICOS (ICOS⁺ cells) but not nontransfected cells (black). **(b)** ICOS mAbs (red) bind to activated CD4⁺ T cells but not resting CD4⁺ T cells. T$_H$1 and T$_H$2 cells stained with mAb 12A8 showed that T$_H$2 cells express higher amounts of ICOS than T$_H$1 cells. **(c)** (Left) ICOS-transfected Jurkat cells were stained with B7RP-1-Ig (red). Staining was blocked with the addition of 12A8 (green). (Right) The approximate IC₅₀ was 0.2 µg/ml for 12A8 (open squares). Despite binding to ICOS, at 10 µg/ml mAb 1C10 did not inhibit B7RP-1-Ig binding (filled squares).

(Fig. 1a). When cells were cultured in the presence of IL-4, ICOS expression was enhanced, whereas repetitive antigenic stimulation in the presence of IL-12 resulted in a down-regulation of ICOS expression (Fig. 1b). The ability of these mAbs to prevent binding of the ICOS ligand B7RP-1 was also assessed. We found that 12A8 inhibited B7RP1- and immunoglobulin (Ig) binding with an IC₅₀ of ~0.2 µg/ml (Fig. 1c). In contrast, 1C10, which stained ICOS-expressing cells, was unable to block B7RP1-Ig binding (Fig. 1c). Neither 12A8 nor 1C10 bound to CD28 transfectants or inhibited B7-1-Ig binding to activated T cells (data not shown). Thus, 12A8 recognized an epitope on ICOS that was common to that used for ligand binding. In contrast, although 1C10 recognized ICOS, it bound to a site that was distinct from B7RP-1.

Cytokine production after ICOS neutralization

We next assessed the role of ICOS in T$_H$ cell function. We determined cytokine production by TCR-transgenic T cells after stimulation with the ovalbumin peptide OVA(323–339), referred to hereafter as OVA, in

vitro or immunization with keyhole limpet hemocyanine (KLH) ex vivo + in vivo treatment with mAbs to ICOS. We found that mAb 12A8 inhibited IL-4, IL-10 production and, to a lesser extent, IL-5 production by CD4⁺ T cells (Fig. 2a). In contrast, the production of the T$_H$1-type IFN-γ was increased after treatment with 12A8 (Fig. 2a). In vivo treatment with neutralizing 12A8 also inhibited IL-4 and IL-10 production (Fig. 2b). The mAb 1C10, which was unable to block B7RP-1-Ig binding, did not alter cytokine release in response to OVA or KLH; these results were not different those obtained with the use of control rat Ig. These data indicate that signaling through ICOS delivers a signal for immune deviation that facilitates T$_H$2 cytokine expression and reduces T$_H$1 cytokine production.

Expression of CD28-B7 family members in lungs

CD28, CTLA-4 and ICOS mRNA expression was measured by Taqman analysis in lung tissue isolated at different time-points during lung allergic inflammation. The inflammatory response to OVA consisted of

Figure 2. Antigen-induced cytokine production after ICOS neutralization. (a) Cytokines were measured in the supernatant of OVA-specific TCR-transgenic spleen CD4⁺ T cell cultures stimulated with OVA peptide (0.3 µg/ml). The neutralizing anti-ICOS mAb 12A8 (black bars) or the nonneutralizing anti-ICOS mAb 1C10 (shaded bars) were added to cultures. Rat Ig (open bars) was used as a control. **(b)** Cytokines in the supernatant of draining lymph node cells collected from KLH-immunized mice after in vivo treatment with 12A8 (closed circles) or 1C10 (open circles) were measured. Rat Ig (open squares) was used as a control.
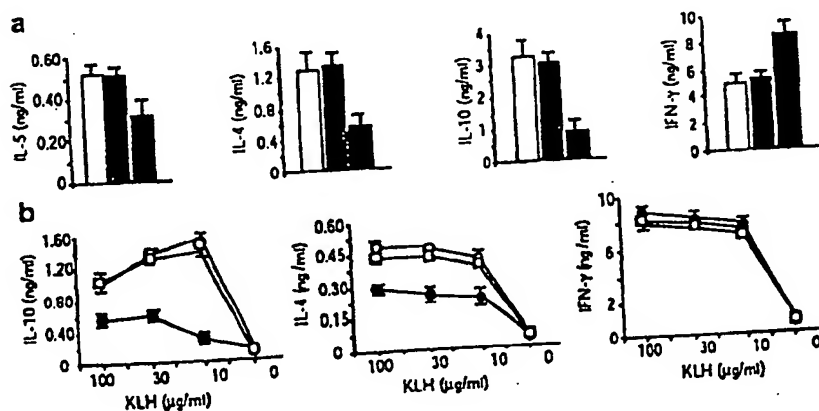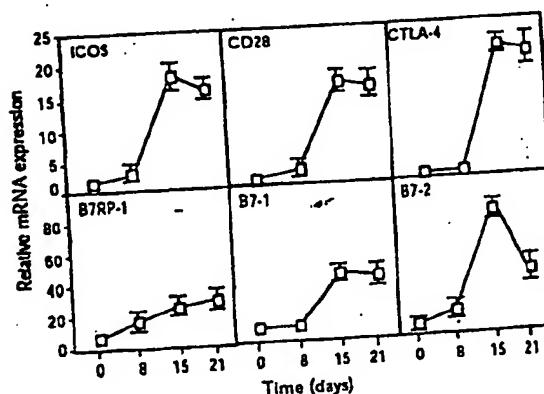
Figure 3. ICOS expression during allergic lung inflammation. Lungs from mice subjected to OVA treatment, 3 h after antigen challenge, on days 0, 8, 15 or 21 were analyzed. Each square represents mean mRNA expression of CD28 and B7 family members from five mice at the time-points indicated.

an accumulation of macrophages in the lung (interstitium and airway lumen) that was maximal in the early stages (3 h after OVA challenge on day 15); the accumulation of eosinophils and lymphocytes reached a plateau in the late stages of the response (3 h after OVA challenge on day 21). CD28, CTLA-4 and ICOS were expressed in low amounts in the lungs on day 0 and day 8 (Fig. 3). However CD28, CTLA-4 and ICOS expression were up-regulated by day 15 of OVA treatment and expression was still maintained by day 21. B7RP-1 was also expressed in the lungs and was maximal by day 21. Similarly, B7-1 and B7-2 mRNA expression was up-regulated by four- and eightfold, respectively, during the course of the inflammatory response (Fig. 3).

To determine the location of ICOS protein expression, lung sections were stained with mAbs to ICOS. No ICOS expression was detected in the lung of PBS-treated mice, but increased expression was observed in the infiltrating parenchymal T cells of OVA-treated animals (Fig. 4a,b). In addition, lymph nodes from mice immunized with KLH were stained with the mAb 12A8 (Fig. 4c–e). The sections showed that ICOS+ cells were present within the B cell follicle after immunization (Fig. 4c). The relationship between ICOS+ cells and germinal center formation was also determined. By day 4 after immunization with KLH, ICOS+ cells could be detected in close proximity to B cells with the early formation of peanut agglutinin–positive (PNA+) germinal centers (Fig. 4d). By day 7, distinct PNA+ cells surrounded by ICOS+ cells could be seen in the lymph nodes (Fig. 4e).

Regulation of mucosal inflammation by ICOS

Leukocyte cell numbers in the lungs of OVA-treated mice were determined 3 h after OVA challenge on day 21. After treatment with mAb 12A8, lymphocyte and eosinophil numbers in the bronchoalveolar lavage (BAL) fluid of OVA-treated mice were reduced by 50% and 70%, respectively (Fig. 5a,b). Comparable suppression was observed with ICOS-Ig. CTLA-4–Ig induced an almost complete reduction in BAL lymphocyte and eosinophil accumulation. As a control, mAb 1C10 was used. The

results obtained with 1C10 did not differ from those obtained with experiments with rat Ig–treated animals or with human Ig, which was used as a control for CTLA-4–Ig and ICOS-Ig. For clarity, only data obtained with 1C10 are shown. These observations were supported by the examination of lung sections, which were assigned a score based on the extent and size of peribronchiolar infiltrates. Animals treated with OVA + the control of peribronchiolar infiltrates. Animals treated with OVA + the control mAb 1C10 had a mean score of 4.7±0.4; administration of 12A8 during OVA treatment decreased the mean score to 2.8±1.1.

To determine whether OVA-induced inflammatory cell accumulation in the lung was prevented rather than delayed by administration of mAb 12A8, the BAL fluid of OVA-treated mice was collected at different time-point on day 21 and eosinophil numbers evaluated. After treatment with mAb 12A8, eosinophil accumulation was reduced at all time-points investigated (Fig. 5c). Similarly, ICOS blockade with 12A8 or ICOS-Ig or CD28 blockade with CTLA-4–Ig abrogated antigen-induced airway hyperresponsiveness (AHR) (Fig. 5d).

Regulation of priming and effector responses

Thus far, our data show that CD28 and ICOS both contribute to T cell–mediated inflammatory responses. We next dissected the contribution of ICOS and CD28 during either priming or effector responses.

Figure 4. ICOS expression in the lung and peribronchiolar lymph nodes during allergic lung inflammation. (a) Sections were prepared from mice on day 21 of OVA treatment and were stained with mAb 12A8. ICOS staining in the lung was indicated by a brown reaction product on a blue counterstain. (b) ICOS expression was undetectable in lungs from PBS-treated controls (a–e). Lymph node sections from KLH-immunized mice were stained with horseradish peroxidase (HRP)–12A8 (brown) and alkaline phosphatase–B220 (blue, to reveal B cells) and showed ICOS+ cells within the B cell follicle on day 7 after immunization. (c). Immunofluorescence staining on day 4 after immunization showed ICOS+ cells (red) associated with B220+ B cells (green) within the follicle and the formation of a germinal center, as revealed by PNA+ cells (blue) (d). Day 7 after immunization showed a germinal center in the lymph node (blue), with B cells stained in green and ICOS+ cells in red (e). Original magnification ×40.
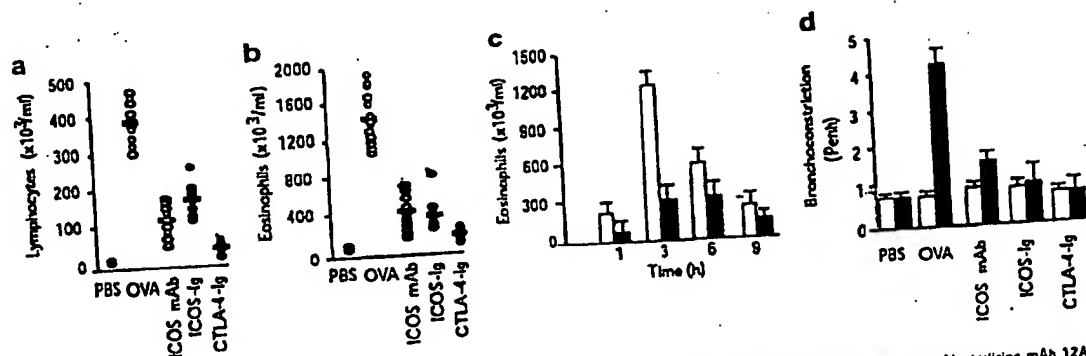
Figure 5. OVA-induced BAL leukocyte accumulation and AHR after ICOS neutralization during allergic lung inflammation. Neutralizing mAb 12A8 (30 μg/mouse, shaded circles), control mAb 1C10 (30 μg/mouse, open circles), ICOS-Ig (100 μg/mouse, blue circles) or CTLA-4-Ig (100 μg/mouse, red circles) were administered before each OVA challenge. Mice exposed to PBS are shown for comparison (filled circles). Accumulation of (a) lymphocytes and (b) eosinophils in the BAL fluid. Each circle represents an individual animal; bars represent the mean for each group. (c) Total number of eosinophils recovered from the BAL fluid of OVA + 12A8-treated mice (filled bars) or OVA + 1C10-treated controls (open bars). Data are the mean±s.e.m. eosinophil numbers (n=10). (d) AHR is shown as are the mean±s.e.m. Penh before (open bars) and after (filled bars) methacholine (MCh) challenge (n=10).

Anti-ICOS (mAb 12A8), ICOS-Ig or CTLA-4-Ig were administered either during priming (on day 0 and 8) or during the efferent response (on day 21 alone). OVA-induced lymphocyte and eosinophil accumulation in the airways (as assessed by analysis of the BAL fluid) were not affected when 12A8 or ICOS-Ig was administered on days 0 and 8. However, when 12A8 or ICOS-Ig were administered on day 21, a 50% reduction in the total number of lymphocytes and eosinophils in the lung was observed (Fig. 6). This indicated that signals delivered by ICOS were important for the activation or recruitment of effector T cells. Changes in the composition of the BAL fluid were associated with corresponding changes in the lung interstitum, as assessed by histology (data not shown). Similarly, CTLA-4-Ig inhibited AHR when administered during priming, whereas ICOS mAb 12A8 or ICOS-Ig inhibited AHR when administered on day 21, when CD28 blockade was not effective (Fig. 6)

ICOS- and CD28-mediated cytokine regulation

To determine whether the differential regulation of mucosal inflammation by ICOS and CD28 could be explained by differentially regulating T cell activation, we measured cytokine production in the BAL fluid after challenge. Treatments, from day 0–21, with both CTLA-4-Ig and mAbs to ICOS suppressed production of IL-4, IL-5, IL-10 and IL-13.

In contrast, IL-12 production was not inhibited by treatment with CTLA-4-Ig, ICOS-Ig or mAbs to ICOS. In response to OVA, no IFN-γ production was detected in the BAL fluid, which indicated that aerochallenge had induced a T_H2 effector response. In addition, treatment with anti-ICOS therapies, which attenuate T_H2 responses, did not result in immune deviation, that is, IFN-γ concentrations were not augmented as a consequence of ICOS blockade (Fig. 7).

Animals were treated with CTLA-4-Ig, ICOS-Ig or mAbs to ICOS either during priming or before the final aeroallergen challenge only, when T_H2 effector cells had been generated. When administered on days 0 and 8, CTLA-4-Ig reduced T_H2 cytokine production; when administered on day 21, it did not regulate IL-4, IL-10 or IL-13 production, although a 50% reduction in IL-5 was observed. In contrast, ICOS blockade on day 21 effectively reduced the production of IL-4, IL-5, IL-10 and IL-13. We also found that ICOS blockade during priming had no effect on IL-4, IL-5 or IL-13 production, although, when administered on days 0 and 8, effective suppression of IL-10 production was observed (Fig. 7).

ICOS-dependent chemokine receptor expression

The contribution of ICOS in the effector phase of the lung mucosal inflammatory response may be, at least in part, explained by a failure to
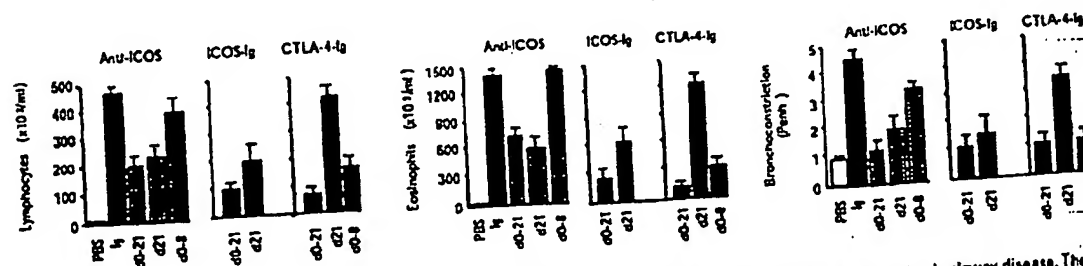

Figure 6. OVA-induced BAL leukocyte accumulation and AHR after ICOS neutralization at different time-points during allergic airway disease. The neutralizing mAb 12A8 (30 μg/mouse), CTLA-4-Ig (100 μg/mouse) or ICOS-Ig was administered via the i.p. route before OVA challenge on days 0, 8 and 15–21 or on days 0 and 8 or day 21 alone.
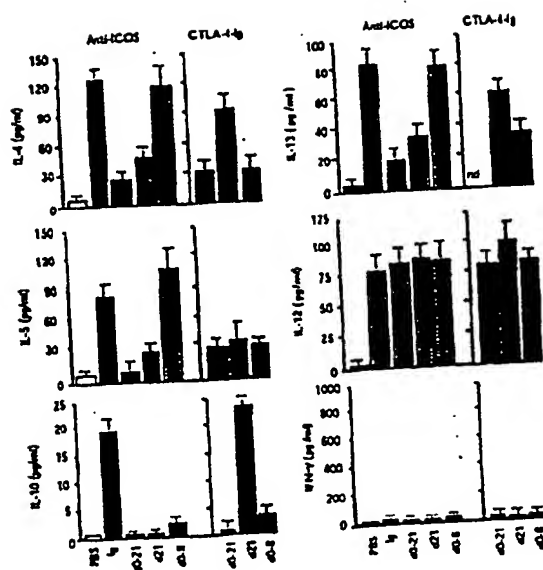
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Figure 7. OVA-induced cytokine production in the BAL fluid after ICOS neutralization during allergic airway disease. Neutralizing mAb 12A8 (30 μg/mouse) and CTLA-4-Ig (100 μg/mouse) were administered via the i.p. route before OVA challenge on days 0,8 and 15–21 or days 0 and 8 or day 21 alone. Data are mean±s.e.m. cytokine concentrations. Data from mice treated with PBS are shown in the open columns, data from control-treated animals are shown the filled columns. Data from ICOS-Ig- or CTLA-4-Ig-treated animals are shown in the shaded columns. nd, not determined.

up-regulate chemokine receptors in the draining lymph nodes. We prepared RNA from the lymph nodes of control Ig-treated or ICOS mAb-treated animals on day 21 before, and 1 h after, allergen challenge. Before allergen challenge on day 21, LNs contained germinal centers and elevated concentrations of antigen-specific IgE were present in the sera. Thus, the animals had mounted an effective response after immunization and aeroallergen challenge. By RT-PCR we determined that, after allergen challenge, CCR3, CCR4 and CCR8 mRNA was increased eight- to tenfold (Fig. 8). Treatment of animals with mAb 12A8 to ICOS prevented the up-regulation of these chemokine receptors. ICOS mAb treatment

also inhibited up-regulation of IL-4 and IL-10 mRNA and increased expression of IFN-γ mRNA in the draining lymph nodes (Fig. 8).

Inhibition of costimulation and regulation of IgE

ICOS⁺ cells can colocalize with B cells in the germinal centers of immunized mice, and ICOS plays a critical role in IL-4 production. Therefore we assessed next the impact of ICOS neutralization on the production of IgE. We found that, when administered on days 0–21, treatment with ICOS mAbs and CTLA-4-Ig abrogated IgE production. A similar reduction in IgE was observed when ICOS and CD28 were inhibited on days 0–8, although ICOS blockade was not as effective as CD28 blockade (Fig. 9).

Discussion

The interactions between CD28 and its ligands B7-1 and B7-2 are required for IL-2 production and T cell clonal expansion[2,3]. In vivo studies with animals deficient in CD28 or B7 genes or with the administration of the B7 antagonist CTLA-4-Ig have shown the essential role played by CD28 in regulating a number of immune responses[22-26]. However, although CD28-dependent mechanisms are implicated in primary T cell responses, secondary immune responses cannot be fully suppressed by administration of CTLA-4-Ig[1,18]. This suggests the existence of alternative or complimentary pathways for effective T cell activation. One possible candidate is ICOS. Through interactions with its ligand B7RP-1, ICOS provides a CD28-independent signal for IFN-γ and IL-4 production, but not IL-2 secretion, in vitro[8-11].

We have shown that in vitro ICOS blockade with a neutralizing mAb reduced expression of IL-4 and IL-10 and augmented secretion of IFN-γ. These data support published data obtained with ICOS-Ig in vitro, which show that ICOS can deliver a signal for immune deviation to Tн2 cytokine production that augments IFN-γ and reduces IL-4 production[9]. Although ICOS regulates Tн2 cytokine production, it is also preferentially expressed on Tн2 versus Tн1 cells, which supports the identification of the gene encoding murine ICOS as a Tн2-overexpressed gene[10]. However, although in vitro ICOS blockade facilitated immune deviation, production of IFN-γ ex vivo was not increased, despite reduced IL-4 and IL-10 secretion. This may be related to the use of complete Freund's adjuvant (CFA) as an adjuvant; because the contribution of ICOS to immune responses has been shown to be influenced by the adjuvant used[13].

Like ICOS, CD28 can promote Tн2 differentiation in vitro[13]. CTLA-4-Ig treatment in vivo can inhibit IL-4 production, but not IFN-γ secretion, during cutaneous leishmaniasis[30] and suppresses IL-4-dependent IgG1 production in a transgenic model of autoantibody production[35]. Similar conclusions were drawn using nonobese diabetic CTLA-4-Ig
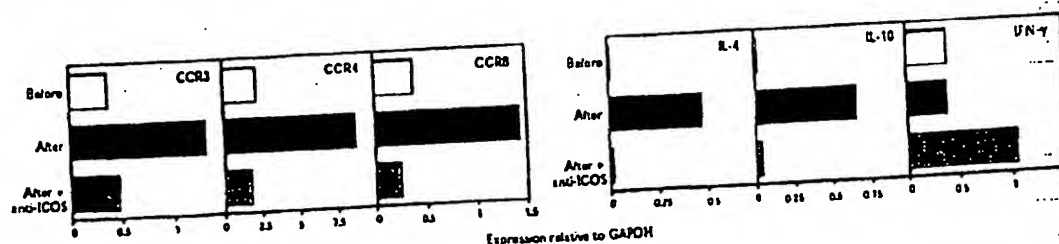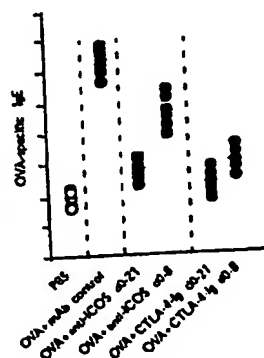

Figure 8. Regulation of chemokine receptor expression in the lymph nodes for ICOS. Lung-draining lymph nodes were removed on day 21, either before or 1 h after antigen challenge of control Ig-treated (open and filled bars, respectively) or anti-ICOS mAb 12A8-treated (shaded bars) animals. CCR3, CCR4 and CCR8 mRNA expression were measured by RT-PCR analysis. Expression of IL-4, IL-10 and IFN-γ mRNA were also evaluated. Each bar represents pooled data from 8–12 animals.

Figure 9. OVA-induced IgE production after ICOS neutralization and treatment with CTLA-4-Ig. Neutralizing mAb 12A8 (30 μg/mouse) or CTLA-4-Ig was administered via the i.p. route before each OVA challenge on days 0.8 and 15–21 or on days 0 and 8, as indicated. Antigen-specific IgE was measured in the sera by specific ELISA. Data are the mean±s.e.m. percentage difference of the OVA-specific absorbance values compared to PBS control animals. Each circle represents an individual mouse.

transgenic mice, which developed enhanced autoimmune diabetes that was associated with enhanced IFN-γ secretion, reduced IgG1 production and diminished T$_H$2 cytokine secretion[?]. These gave rise to the general concept that T$_H$2 responses are more dependent on costimulation than T$_H$1 responses. Thus, in some respects, CD28 and ICOS have overlapping functions that regulate the outcome of immune responses. However, CD28- and ICOS-mediated signaling differ in some important respects.

Although CD28 and ICOS both inhibit T cell expansion, CD28-mediated signaling is uniquely required for IL-2 production, whereas ICOS delivers a signal for secretion of tumor necrosis factor-α and IL-10 after superantigen administration[?]. In addition, during experimental allergic encephalomyelitis (EAE), ICOS-deficient mice exhibit markedly exacerbated responses[14], whereas CD28- or B7-deficient animals have attenuated disease[?]. The underlying mechanism(s) by which these molecules have opposing phenotypes in EAE is unclear, although one possible explanation is consistent with the ability of ICOS to facilitate T$_H$2 differentiation associated with reduced IFN-γ production. However, we have observed that, like the data obtained with ICOS-deficient mice, early therapeutic intervention increases clinical disease, which is associated with increased IFN-γ. Delayed treatment with mAbs to ICOS markedly impairs the onset of clinical symptoms during EAE, which is associated with reduced IFN-γ production[?]. Thus, the antigen experience of the T cell appears to be a critical factor in determining the outcome of ICOS blockade. Although ICOS engagement enhanced T$_H$2 and reduced T$_H$1 responses during differentiation, ICOS can regulate the production of both IL-4 and IFN-γ by recently activated T$_H$ effector cells[10]. In addition, the nature of the immune response may also influence the role of ICOS. This is illustrated in another T$_H$1-mediated model where inhibition of ICOS, either with a blocking mAb or with the use of ICOS-deficient mice, prolongs rejection of cardiac allografts and is associated with reduced IFN-γ and IL-10 production[?].

To further address whether CD28 and ICOS have distinct or complimentary roles in regulating T$_H$2 responses, we next used a model of allergic lung inflammation driven by inhaled allergens[?]. The development of a lung eosinophilic inflammatory response is dependent on T cell–derived cytokines, including IL-4, IL-5 and IL-13[?]. CTLA-4-Ig, as well as ICOS blockade, inhibited T cell expansion and/or recruitment and reduced the recruitment of eosinophils to the lungs. These data support work by several investigators, who used either B7- or CD28-deficient animals or administered CTLA-4-Ig[?,?,?], and show that ICOS signaling is also critical in this process.

Although antigen-inexperienced CD4$^+$ T cells require B7-mediated signaling for IL-2 production, clonal expansion and the acquisition of effector function, optimal activation of recently activated T$_H$ cells is B7-independent[?]. In addition, reactivation of memory cells occurs independently of both B7-1 and B7-2[41]. Our data show marked differences in the requirement for ICOS versus CD28 engagement. Blockade of CD28 interfered with priming, but ICOS contributed to effector responses. Whether there are also distinct subpopulations of cells that secrete different patterns of cytokines that use either ICOS and/or CD28 remains to be determined.

The reduced cytokine concentrations obtained from the BAL fluid during the effector response may have been due to the failure of the cytokine-producing cells to migrate from the secondary lymph nodes to the lungs, a process regulated by chemokines and their receptors[?]. Our data on chemokine receptor expression raises the possibility that costimulatory signals regulate receptors that control the migration of effector/T$_H$P cells to sites of allergic mucosal inflammation. The relationship between ICOS-mediated costimulation and the induction of chemokine receptors such as CXCR5 and CCR7, which distinguish between follicular T$_H$ cells[?,?] and central or effector memory cells[?], remains to be determined.

ICOS-deficient mice are unable to generate germinal centers after protein immunization because of their impaired ability to up-regulate CD40 ligand[?]. ICOS$^+$ CD4$^+$ T cells were present within the B cell follicles of immunized and challenged, but not PBS-exposed, mice. This raises the possibility that ICOS$^+$ T cells receive signals through B7RP-1–expressing B cells and acquire the capacity to produce IL-4. In turn this would regulate the B cell class switch to IgE. In this context, ICOS blockade reduced IgE expression, although ICOS makes a smaller contribution to this process than CD28 does. Our data support published data obtained with ICOS-deficient mice that have a reduced ability to produce IgG1, IgE and also IgG2a after immunization with T-dependent B cell antigens[?,?] but produce normal antibody titers after immunization with T-independent antigens[?]. However, although ICOS plays a critical role in Ig production after immunization with soluble antigen alone or antigen in alum or incomplete Freund's adjuvant, the immune response to antigen in CFA is ICOS-independent[?]. Similarly, after infection with the nematode Nippostrongylus braziliensis, ICOS fails to regulate Ig secretion[?]. The precise basis for these differences is undetermined, but clearly the nature and/or strength of the immune stimuli greatly influence the dependence on ICOS-mediated costimulation.

We propose, therefore, the following model for the regulation and coordination of T cell-dependent inflammatory responses by CD28 and ICOS. T cells first become primed by a CD28-B7-dependent mechanism. Ligation of CD28 also up-regulates the chemokine receptor CXCR5[?], which allows these primed T$_H$P cells to migrate to the edge of the B cell rich follicle. Here they encounter antigen-bearing B cells that have also now accumulated in this area. These B cells then present peptides to primed antigen-specific T cells and, through B7RP-1, deliver a different signal that specifically facilitates T$_H$2 cytokine production. In turn, this up-regulates CD40 ligand, which delivers cognate signals to favor B cell expansion. B7RP-1-ICOS signaling could provide the proposed "step two of signal two"[?]. It would occur upon encounter with antigen-specific B cells that had endocytosed the same antigen for which the primed T$_H$P cells are antigen-restricted, thus ensuring the specificity of the immune response.

In addition to regulating this step of the immune response, B7RP-1-ICOS interactions would also deliver a signal for the up-regulation of CCR3, CCR4 and CCR8. As the ligands for these receptors—eotaxin,

MDC and I-309—are induced during allergic inflammation from epithelial cells and monocytes and have been implicated in mediating T_H2 accumulation in the lungs[...], ICOS-mediated costimulation of effector cells would then provide the signal for these effector cells to migrate from secondary lymph nodes into the airways. These data also provide evidence that suggests inhibition of signaling *via* the B7RP-1-ICOS pathway may represent a new target for the treatment of diseases such as allergic asthma, which are characterized by inappropriate T_H2 activation, without compromising host protective responses to pathogenic bacteria and viruses.

Methods

Monoclonal anti-ICOS generation. Why rats were immunized via the intraperitoneal (i.p.) route with purified murine ICOS-Ig (100 μg) in CFA and boosted via the i.p. and subcutaneous routes. Splenocytes were fused with SP/2 myeloma cells and the resulting clones were screened for binding to ICOS-transfected Jurkat cells. Positive clones (12 of 600) were subcloned by limited dilution and isotyped with isotyping secondary antibodies (PharMingen, San Diego, CA). Identification of neutralizing mAbs were done by preincubating ICOS-transfected Jurkat cells (1×10⁴/200 μl) with anti-ICOS (10 μg/ml) at room temperature for 30 min and then adding B7RP-1-Ig (2 μg/ml) to the mixture for another 30 min. ICOS-B7RP-1-Ig binding was detected by FACS with a phycoerythrin (PE)-conjugated goat anti-human Ig (PharMingen).

In vitro OVA stimulation. Mice expressing the transgene encoding the DO11.10 αβ TCR, which recognizes chicken OVA amino acids 323–339 in association with I-A^d, were provided by D. Loh (Washington University, St Louis, MO). OVA-specific TCR-transgenic CD4⁺ T cells were isolated from the spleen with negative-selection columns (R&D, Minneapolis, MN). Antigen-presenting cells (APCs) were prepared from mitomycin C-treated (50 μg/ml, 30 min) BALB/c splenocytes. OVA-specific CD4⁺ T cells (2×10⁶/ml) were stimulated with APCs (2×10⁶/ml), the peptide OVA(323–339) (0.3 μg/ml), anti-ICOS (mAb 12A8 or 1C10, 10 μg/ml) or control rat Ig (10 μg/ml) for 5 days, washed and aliquoted into 96-well plates. Activated CD4⁺ T cells (2×10⁶/well) were restimulated with fresh APCs (2×10⁶/well) and OVA (1 μg/ml) for another 2 days. The supernatants from these cultures were then collected and tested for cytokine production.

KLH antigen immunization. BALB/c mice that were 6–8 weeks old (Jackson Laboratory, Bar Harbor, ME) were immunized with KLH (1 mg/ml), emulsified in CFA (v/v 1:1), via the left footpad and base of the tail (50 μl/site). After 10 days, the draining lymph nodes were removed and cells were stimulated with KLH or, as a control, medium. The supernatants from these cultures were collected 48 h later and tested for cytokine production. Lymph nodes cells were removed after immunization with KLH for immunohistology on day 4 and day 7.

Induction of mucosal inflammation in vivo. C57BL/6J mice that were 6–8 weeks old (Jackson Laboratory) were housed in a specific pathogen-free mouse facility. The mouse model of lung inflammation used consisted of a sensitization phase (0.1 mg/mouse of i.p. OVA on day 0) (Sigma, St Louis, MO) and an induction of the response phase (2% OVA for 5 min by aerosol on day 8 and 1% OVA for 20 min by aerosol on days 15–21). PBS (i.p. and/or aerosol) was administered to mice as a negative control. For the neutralizing experiments, mice also received 30 μg/mouse of anti-ICOS or 100 μg/mouse of ICOS-Ig or CTLA-4-Ig. Antibodies and fusion proteins were administered i.p. 30 min before OVA challenge on days 0, 8 and 15–21 or on days 0 and 8 or on day 21. OVA-treated control mice were injected with the same amount of control mAb at the same time-points as were administered during treatment. Three hours after OVA administration on day 21, mice were killed by CO₂ asphyxiation and lung leukocyte accumulation analyzed by bronchoalveolar lavages. AHR, expressed as enhanced pause (Penh) was measured 3 h after the last antigen challenge by recording respiratory pressure curves with whole-body plethysmography (Buxco Technologies, Sharon, CT) in response to inhaled methacholine (MCh, Aldrich, Milwaukee, WI) as described[...].

Measurement of gene expression by real-time PCR analysis. Total RNA from the lungs of OVA-treated mice or control littermates was extracted by single-step method with RNA STAT-60 (Tel-Test, Friendswood, TX). ICOS, CD28, CTLA-4, B7-1, B7-2, and B7RP-1 expression profiles were determined by RT-PCR (Taqman™, Perkin-Elmer, Norwalk, CT). Briefly, an oligonucleotide probe was designed to anneal to the gene of interest between two PCR primers. The probe was then fluorescently labeled with FAM (reporter dye) on the 5′ end and TAMRA (quencher dye) on the 3′ end. A similar probe and PCR primers were designed for GAPDH. The probe for this gene incorporated VIC as the reporter dye. PCR reactions were run that included the primers and probes for these two genes, as well as cDNA made from various cells and tissues. As the polymerase moved across the gene during the reaction, it cleaved the quencher dye from one end of each probe, which caused a fluorescent emission that was measured by the Sequence Detector 7700. The emissions recorded for each cDNA could then be converted into the amount of expression for the gene normalized to the expression of GAPDH. In addition,

lung draining lymph nodes were removed on day 21 before and 1 h after antigen challenge, cDNA prepared as described and the amounts of CCR3, CCR4, CCR8, IL-4, IL-10 and IFN-γ determined. Animals were treated with anti-ICOS or Ig control 1 h before challenge.

Assessment of ICOS protein by immunohistochemistry. Protein expression was determined in noninflamed and inflamed mouse lung tissue samples using the mAb to ICOS and a modified biotin-avidin-staining method. ICOS, B cell and PNA⁺ cell expression in the lymph nodes from KLH-immunized animals was also determined. Frozen sections were stained with anti-ICOS, then biotinylated anti-rat Ig and HRP-streptavidin. B cells were labeled with biotinylated B220 and detected with alkaline phosphatase streptavidin and germinal centers detected with biotinylated PNA and avidin (both from Vector Laboratories, Burlingame, CA). All washes between antibody incubations were done in PBS Tween, and slides were mounted for microscopy in Fluoromount-G (Southern Biotechnology, Birmingham, AL).

Cytokine and IgE measurement. Serum and supernatant cytokine release was determined by ELISA. Sera from five different mice were analyzed in each group. Serial dilutions of samples were analyzed for IL-4, IL-5, IL-10, IL-12, IL-13 and IFN-γ expression with commercial ELISA kits (Endogen, Boston, MA).

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